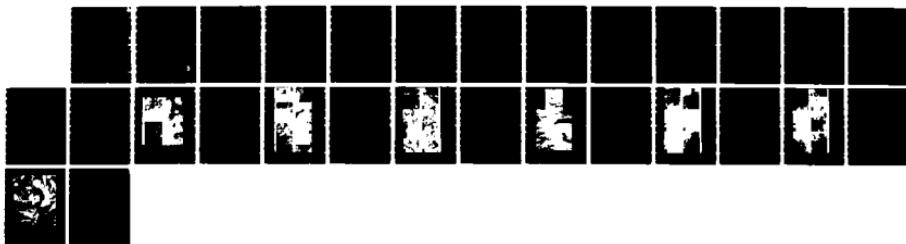
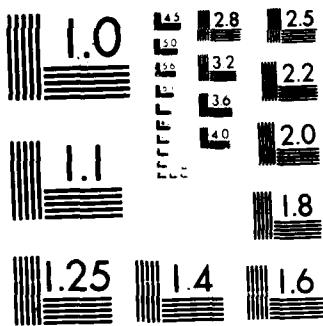


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TISSUE CULTURE AND IN VIVO MODELLING OF CORNEAL
OPACIFICATION AND OCULAR INJURIES INDUCED BY
PULSED MILLIMETER WAVES

Annual and Final Report

John R. Trevithick, Ph.D.

February, 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

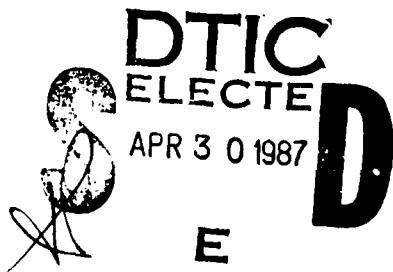
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) ►The eventual aims of these experiments were to use intact corneas incubated in vitro and corneas of rats or rabbits exposed in vivo to high energy pulsed millimeter waves to study the development of corneal damage. Such experiments were expected (1) to establish conditions for corneal damage and (2) to elucidate the mechanisms by which the damage occurs. In order to perform these tests, studies of damage to corneas exposed to elevated temperatures in vitro are required as controls to establish a baseline. continued... / / /		

→ Rabbit corneas were exposed for a period of 30 minutes to elevated temperatures.(35.5 as control, 37, 39, 40, 42, 45 and 50°C). Corneas were suspended either in phosphate buffered saline or in medium 199 with 10% fetal calf serum. Following incubation corneas were removed and fixed in Karnovsky's fixative at 4°C for 24 hr, then transferred to cacodylate buffer prior to examination by scanning electron microscopy. Generally approximately one hour elapsed between the time the eyes were removed and corneal incubation began. Epithelial cell changes appeared to be more pronounced than endothelial cell changes at all temperatures. Epithelial cells changed from flat cells with elevated intercellular junctions and short cylindrical microvilli at 37°C, apparently swelling and losing their microvilli progressively at 39°C, until at 42°C some cells were missing leaving denuded areas and few microvilli were visible; at 45 and 50°C increased damage was seen, including cell curling to look like potato chips, increased stromal denudation and fibrous cell surfaces. Both these changes and endothelial changes (below) were accentuated in vitamin E containing medium. For endothelial cells also, progressive increases in damage were seen as temperature increased: "potato chip" cells, occasional large processes (39°C), some cell swelling obscuring cell boundaries, more large processes (42°C), clumps and cell processes covering cell surfaces (45°C) leading to rough fibrous cell surfaces, with many holes, after exposure to 50°C. Stromal changes, leading to stromal disorganization and separation into many fine individual strands at 50°C also occurred with increasing temperature.

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FOREWORD

A. List of Professional Personnel Employed on This Project

Principal Investigator	Dr. John R. Trevithick, Ph.D.
Research Associate	Dr. Margaret O. Creighton, Ph.D.
Technician (Part-time)	Dr. Peter Galsworthy, Ph.D.
	Mrs. Andrea Hannington

B. Animal Care

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH)78-23, Revised 1978).

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INTRODUCTION

Although millimeter wave radars are now strategically important, only one study of the effect of millimeter waves on the cornea has been reported (Rosenthal et al., 1975). This study did not use the pulsed mode of millimeter waves which is commonly in use in such radars. Because we have discovered apparent differences between similar doses of pulsed and CW microwaves (Stewart-DeHaan et al., 1980) in preliminary experiments and because we have succeeded in separating the effect of heating from the effects due to the electromagnetic field, for microwaves, we wished to devise a similar system for irradiation of the cornea *in vitro* which would offer similar advantages for the study of millimeter wave damage to the cornea.

The first step in these experiments was to devise appropriate media and conditions for the tissue culture of corneas, to be used for the experiments investigating their exposure to millimeter waves. The second step, also described in our Annual Report, June 1981 under DAMD17-80-G-9480, was to incubate the cultured corneas at different elevated temperatures in order to investigate the effect of incubation at elevated temperature on the cornea. The third stage, reported in our Annual Report, June 1982 under DAMD17-80-G-9480 and DAMD17-82-C-2018, was to test the effect of exposing incubated corneas to non-ionizing radiation, for which we selected ultraviolet as convenient and accessible in our laboratory, since the appropriate millimeter wave irradiation apparatus was not yet operating. In 1983 the millimeter wave irradiation apparatus was functioning, permitting us to perform preliminary experiments on rabbits *in vivo* which were reported in our Annual Report, June 1983 (DAMD17-82-C-2018).

These experiments dealing with the corneal sensitivity of environmental temperature are of general interest as well as military interest, as discussed in the following (from our paper submitted for publication).

The eye is exposed to many potentially dangerous and noxious environmental agents, and to infrared and ultraviolet radiation. Because of its position at the surface of the eye, the cornea is responsible both for the major refraction of light to be focussed on the retina and is also the first part of the eye to encounter such noxious agents and radiation. It is in intimate contact with the environment, protected only by a thin layer of liquid and a monolayer of peculiar lipids - the Meibomian lipids (Tiffany, 1978; Tiffany and Marsden, 1980). Survivable environmental temperatures on earth can reach over 50°C, and such temperatures are also associated with high levels of infrared and ultraviolet radiation in deserts, which appear likely to affect the cornea.

Several studies have been performed of the effects of infrared radiation on the cornea (and other eye components) (Pitts, Cullen and Dayhaw-Barker, 1980; Bergeron, Farrell, Green and McCally, 1981), and on corneal innervation (Simkovia, 1982); ultraviolet radiation also has been shown to have damaging effects on the cornea, inducing lysosomal changes (Cullen, 1980a), which could be shown to be additive (Cullen, 1980b).

Studies have been performed showing corneal nerve changes induced by temperature (Simkovia, 1982) and corneal responses to elevated temperature following thermokeratoplasty (Arentsen, Rodriguez and Laibson, 1977); Handelberg, Rao and Aquavella, 1980), and even chromosomal aberrations induced

in Chinese hamster corneas following microwave irradiation (Yao, 1978). Unfortunately, no systematic study of the effect of elevated temperature on the structure of corneal epithelial and endothelial cells and stroma seems to have been performed.

Because we had previously performed such a study of the response of the isolated lens to elevated temperature (Stewart-DeHaan, Creighton, Ross, Trevithick, 1981), we felt it would be appropriate to extend this approach to investigating the effect of elevated temperature on the cornea. In these studies we chose to use the technique of scanning electron microscopy to obtain an overview of the effects of such elevated temperatures on the cell surfaces, to facilitate the visualization both of cell detachment from the stroma at elevated temperatures and of stromal swelling, and to evaluate whether vitamin E might exert a protective effect similar to that found for heat (Stewart-DeHaan et al., 1981), and diabetic cataractogenesis in the lens (Ross, et al., 1983).

MATERIALS AND METHODS

Eyes of pigmented rabbits were obtained at a nearby slaughterhouse, placed in room temperature medium-either PBS or M199 depending on the experimental treatment and brought to the laboratory within less than half an hour. The eyes were rinsed six times in sterile phosphate-buffered saline (PBS) at room temperature so as to remove any bacteria. If any bacteria or fungi remained, their growth was inhibited by the antibiotics (penicillin (mg/ml), streptozotocin (mg/ml) and fungazone (mg/ml)) added to the M199 medium used for the corneal incubations (Buck, 1979).

For incubation, the corneas were removed from the eyeball by trimming around the edge of the cornea using iris scissors (Buck, 1979), and placed in a closed plastic tube, diameter 1.7 cm (Falcon #2059) containing 5.0 ml PBS or M199 respectively, depending on previous treatment, at specific temperatures (37° , 39° , 42° , 45° , 50°) for 30 min exactly. Corneas generally were picked up at approximately 10 am and preparation of corneas was complete by approximately 11 am. Five corneas were used for each incubation condition. Following such exposure, the corneas were removed and placed in Karnovsky's fixative at 4°C then placed in a refrigerator and held overnight (24 hr). The fixative was then replaced by 0.1M sodium cacodylate buffer and the sample processed as usual for SEM (dehydration, critical point drying and sputter-coating (Creighton et al, 1978). Scanning electron microscopy was performed using a Hitachi HHS-2R or S-650 scanning electron microscope.

RESULTS

Scanning electron microscopy revealed striking changes from normal corneal morphology as temperature of the buffered saline or Medium 199 increased. Similar changes were noted in both incubation liquids. These changes, summarized diagrammatically in Figure 1, were detected by scanning electron microscopy and are shown in figures 2-8.

Epithelial cells appeared normal at 35.5°C (Fig. 2a) with intercellular junctions appearing as a ridge and the cell surfaces covered by short stubby cylindrical microvilli. This is maintained at 37°C (Fig. 3a). After 30 minutes at 39°C , some cells appear to have swelled, leaving occasional troughs at intercellular junctions, and at the cell surface there are disorganized

microvilli which were elongated and intertwined cylinders (Fig. 3b). After 42°C (Fig. 4a), the epithelial cells had torn apart and some were missing from the corneal surface, leaving denuded areas, while microvilli were sparse and appeared only as small bumps on the surface. After exposure to 45°C and 50°C, cells progressively showed more damage with increasing temperature, curling up to look like potato chips with almost complete loss of microvilli on the cell surface, then apparently dying and falling off, leaving large areas of the stromal surface exposed and cell surfaces fibrous and pitted at 50°C (Fig. 4c).

At elevated temperatures, progressive changes involving nuclei of epithelial cells appeared to occur. In initial stages of damage (Fig. 5), the cell surface above the nuclei, which in normal cells could barely be distinguished from that over the cytoplasm, began to elevate centrally as the nucleus apparently contracted and became approximately spherical or egg-shaped. These cells looked like fried eggs in which the nucleus was the yolk. A presumed next stage in this degeneration is seen in Fig. 5d, in which the nuclear contents were apparently extruded through the cell surface in an egg-shaped mass. A further stage in the process is illustrated in Fig. 3c, which shows cells with "potato-chip" boundaries which have only a hole where their nucleus should have been located.

Endothelial cell damage similarly appeared to occur progressively as temperature increased (Fig. 6,7). At 37°C (Fig. 6a) and 39°C (Fig. 6b) cell edge effects increased, resulting in curled-up cells in corneal border regions as the temperature increased. As well, occasional processes larger than normal could be seen protruding from the cell surface.

At 42°C and above (Fig. 7a), areas of the endothelial surface had swelled, obscuring cell boundaries, and the cell surfaces had become rough and undulating. There were more numerous large processes protruding from the cell surfaces. After exposure to 45°C (Fig. 7d) numerous clumps and cellular processes dotted the endothelial corneal surface. After exposure to 50°C (Fig. 7e), the endothelial cells surfaces had become fibrous and rough, with a few small holes. The areas of cell-cell attachment at the cell boundaries appeared to be less tight and gaps between cells could be seen.

Exposure of the stroma (Fig. 8) by a perpendicular cut through the cornea indicated increasing disorganization and disappearance of cells from the stroma as temperature increased. While at 37°C and 39°C large supercables consisting of multiple strands were visible, at about 42°C they showed greater disorganization and separation into many fine individual strands.

It was noted that progressive damage also occurred with increasing time of incubation at 39°C. At short times of incubation cell swelling occurred in the peripheral, but not central area of the cornea, and cell borders appeared depressed when compared to their normal appearance. After 45 min at 39°C in PBS, extensive swelling had occurred with denudation of epithelial cell surfaces.

Comparison of Vitamin E-Treated with Untreated Corneas At Different Incubation Temperatures in Medium 199

Surprisingly, addition of vitamin E (2.4 M) to the incubation medium appeared to result in significantly more damage in both epithelial and endothelial cells at all temperatures. For epithelial cells, little difference was observed at 35.5°C (Fig. 2c). In contrast to the normal appearance of cells exposed to 37°C (Fig. 2d), after vitamin E treatment occasional cells appeared to have a "potato-chip" appearance and some cells appeared to have loosened from the monolayer (Fig. 3d). At 39°C most untreated cells had relatively normal appearance (although some intercellular junctions and microvilli had changed (see above)), but most vitamin E-treated cells were either swollen or exhibited a "potato-chip" appearance (Fig. 3e). In an additional experiment at 40°C, epithelial cells showed some areas denuded of cells, and other areas in which cells were swollen, but otherwise, areas of fairly normal cells could be observed (Fig. 3f). By contrast, all vitamin E-treated cells were observed to be swollen with especially obviously swollen nuclei, and cells were seen breaking apart from each other. At 42°C damage was more severe and areas of epithelial denudation appeared larger in vitamin E-treated than in untreated corneas (Fig. 4d). By 50°C, almost complete removal of epithelial cells from vitamin E-treated corneas occurred while many cells could still be observed in untreated corneas, although these often were curled up or had "potato-chip" appearance and appeared to be in the process of coming off (Fig. 4f).

Although endothelial cells appeared to be more stable, similar effects of vitamin E were observed in apparently potentiating the damage caused by elevation of temperature. Although at 39°C and 40°C, cell boundaries appeared to be somewhat looser with occasional spaces, after vitamin E treatment the cells appeared slightly swollen and some broader areas were even denuded of cells (Fig. 6e,f). By 45°C, similar morphological effects were observed for untreated cells (to those observed at 40°C (in vitamin E treated corneas), while vitamin E-treated cells were devoid of border interdigititation with dull, flat surfaces completely lacking microvilli (Fig. 7e). By 50°C the appearance varied: small areas were denuded, a few areas had rough and fibrous cell surfaces with pits and holes, other untreated cells had a flattened appearance with no microvilli and were swollen, with cell borders; some cells were breaking apart, while in E-treated corneas larger areas were denuded, and the remaining cells appeared to be breaking apart from each other and to exhibit a fine granularity, reminiscent of matt finish paint, on their exposed surfaces (Fig. 7f).

DISCUSSION

The changes in the cornea at elevated temperature appeared to occur progressively with both time and increasing temperatures. The degree of damage noted in the incubations reported here appeared to result in slightly more extensive damage to the corneal epithelium than to the endothelium. The incipient changes: curling up "potato-chip" appearance and exfoliation of epithelial cells, begin to occur visibly at somewhat lower temperature (42°C) than that found for the endothelial surface, although for the cell surfaces, holes and fibrous appearance both were found to a similar extent at 50°C. Such changes are consistent with the important and well-known role of the endothelium in maintaining the dehydration of the cornea. After exposure to

50°C for 30 min, the damaged endothelial cells are probably not able to perform this function, leading to the swelling of the stroma and disruption of the regular stromal fibrous structure found in Fig. 8.

Similar accumulative effects on the cornea have been observed by Cullen (1980) for ultraviolet irradiation. For infrared radiation by laser, Bargeron, Farrell, Green and McCally (1981) reported endothelial temperature increases as high as 50°C . These studies indicated that rabbit endothelial cells in vivo are not damaged until exposure durations ten times or more longer than the exposures which produce epithelial damage, although the temperatures for damage thresholds to either are approximately identical.

Usually in damaged corneas, it is known that the epithelial cells migrate to cover denuded areas where cells are lost. Since these corneas were incubated in PBS or Medium 199, it is likely that any cells not adhering tightly would be lost in the medium.

The changes in the cell nucleus are consistent with a super-contraction of the cellular actin, a substantial portion of which is located in the nucleus. Further experiments are underway to investigate this possibility by specific fluorescent stains for actin. Such a supercontraction and the "potato-chip" appearance of such cells are consistent with an explanation involving changes to the cytoskeleton, which is responsible for maintaining the cell's normal edge extension and flattening. If changes in ATP or adenylate energy charge occurred as a result of inactivation of the mitochondrial enzyme systems responsible for producing ATP, this could in turn influence the integrity both of actin and other cytoskeletal elements.

These studies do not define the biochemical events in elevated temperature stress of corneas, which still need to be investigated. The mechanisms involved may involve inactivation of one or several enzyme system, such as $\text{Na}^{+}\text{K}^{+}$ -ATpase, or some cytoskeletal element such as actin or tubulin which is sensitive to elevated temperature. The differential expression of proteins in epithelial cells when compared to endothelial cells may explain the apparent higher sensitivity of the corneal epithelium to elevated temperature.

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Figure 1

Diagram summarizing changes in rabbit corneas exposed to elevated temperatures: incubations of isolated corneas were performed in vitro in Medium 199 for 30 minutes. Corneas were fixed immediately in Karnovsky's fixative and prepared for scanning electron microscopy as described in text. The changes referred to in these diagrams are illustrated by scanning electron microscopy in figures 2-8 following.

Figure 1

E P I T H E L I A L		E N D O T H E L I A L	
- VIT. E	+ VIT. E	- VIT. E	+ VIT. E
NORMAL RIDGES	NORMAL	NORMAL	NORMAL
35.5° C			
NORMAL	"POTATO CHIPPING" NORMAL LOOSE	NORMAL	NORMAL
37° C			
MOST NORMAL, RIDGES LOST microvilli short though some holes.	MOST CELL SWOLLEN OR "POTATO CHIPPING"	NORMAL (PERHAPS LOOSER CELL BORDERS?)	
39° C			
Denuded areas, cells swollen in areas but still area of fairly normal cells (center)	All cells breaking apart from each other, swollen, especially nuclei.	Borders are looser	Are cells slightly swollen? Sore areas denuded.
40° C			
denuded areas, cells breaking apart from one another	whole areas bare of cells		
42° C			
all cells swollen great areas denuded		May be swollen some areas denuded	cell borders devoid of indigitation now are very flat and dull looking
45° C			
still lots of cells but curled and coming off	maybe down to stroma in places almost seems devoid of cells	swollen, cell borders under strain some breaking apart	cells breaking apart from each other and becoming fragile
50° C			

Figure 2

Changes in corneal epithelial and endothelial cells visualized by SEM as temperature of incubation of corneas in Medium 199 increases. Corneas were fixed after 30 mins incubation at control temperature of 35.5°C, critical point-dried and sputter coated as described in the text.

- a) Epithelial cells incubated at 35.5°C without vitamin E in Medium 199
- b) Endothelial cells incubated at 35.5°C without vitamin E in Medium 199.
- c) Epithelial cells incubated at 35.5°C with vitamin E (2.4 M) in Medium 199.
- d) Endothelial cells incubated at 35.5°C with vitamin E (2.4 M) in Medium 199.

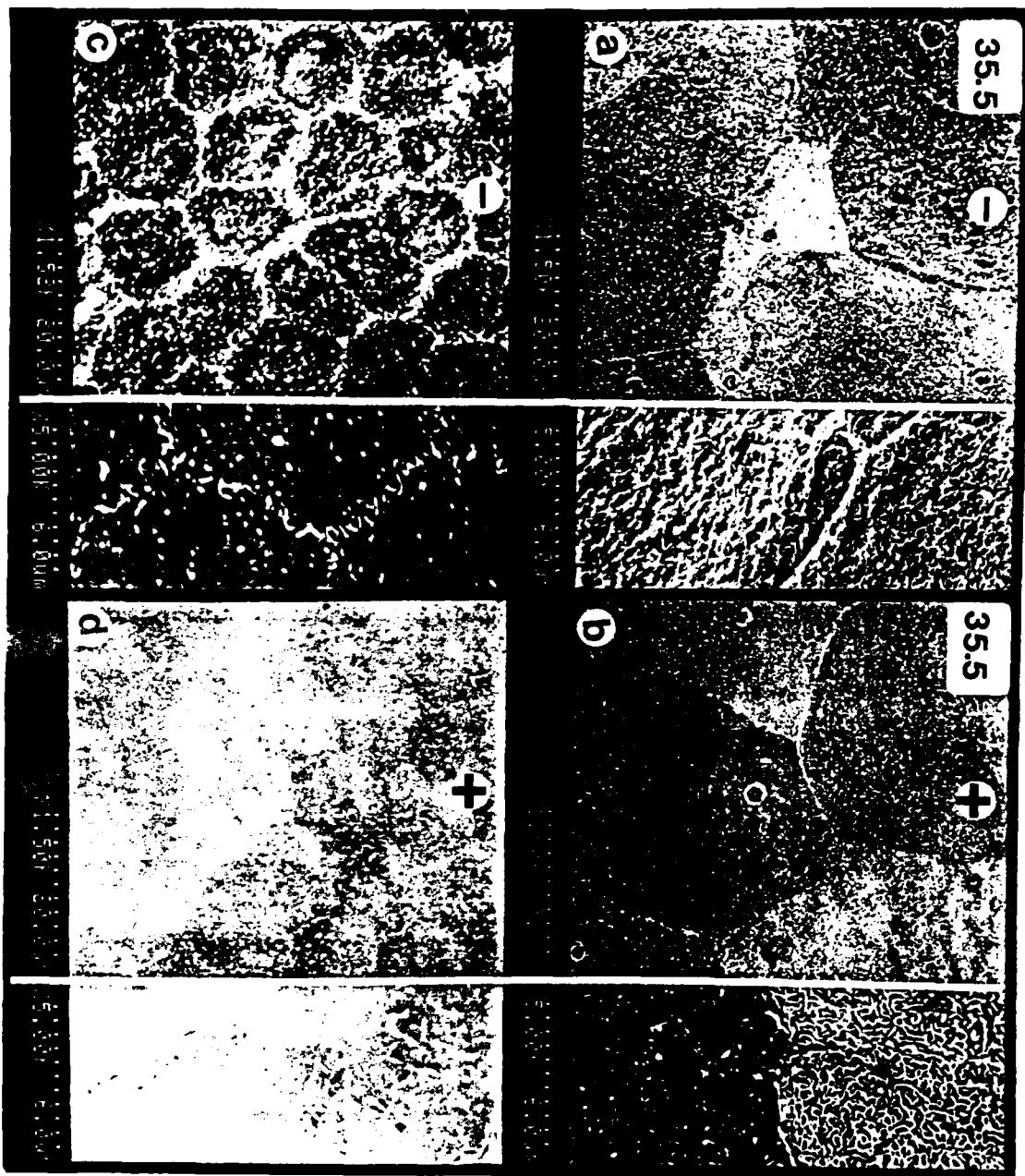


Fig. 2. Changes in corneal epithelial and endothelial cells visualized by SEM as temperature of incubation of corneas in Medium 199 increased.

Figure 3

Series of pictures showing changes in corneal epithelial cells visualized by SEM as temperature of incubation of corneas in M199 is increased from 37° C to 40° C.

a),b),c) are of cornea epithelial cells incubated 30 min without vitamin E added to the medium.

d),e),f) are of cornea epithelial cells incubated 30 min with vitamin E (2.4 M) added to the medium.

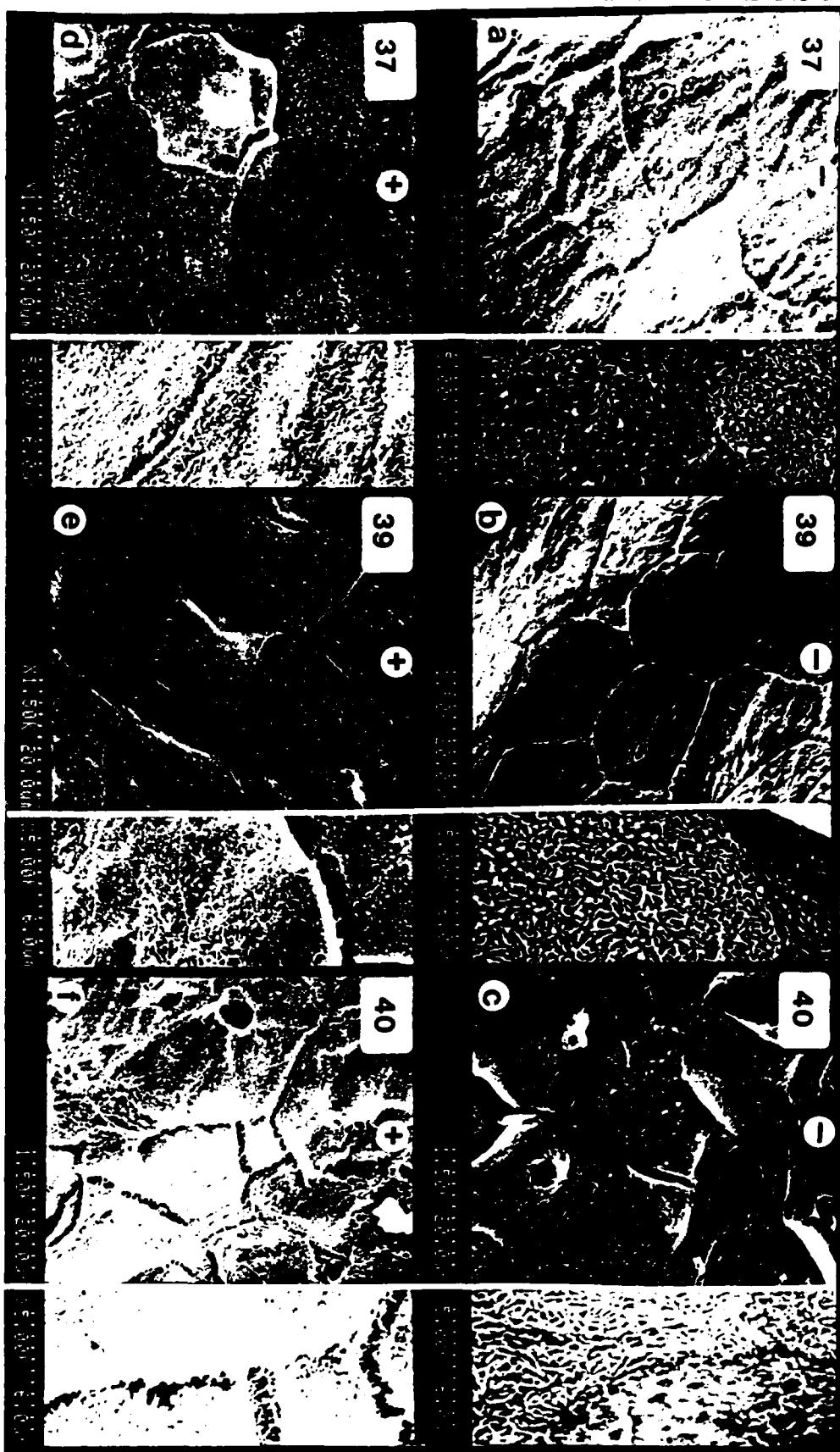


Fig. 3. Series of pictures showing changes in corneal epithelial cells visualized by SEM as temperature of incubation of corneas in M199 is increased from 37°C to 40°C.

Figure 4

Series of pictures showing changes in corneal epithelial cells visualized by SEM as temperature of incubation of corneas in M199 is increased from 42°C to 50°C.

a),b),c) are of cornea epithelial cells incubated 30 min without vitamin E added to the medium.

d),e),f) are of corneal epithelial cells incubated 30 min with vitamin E (2.4 M) added to the medium.

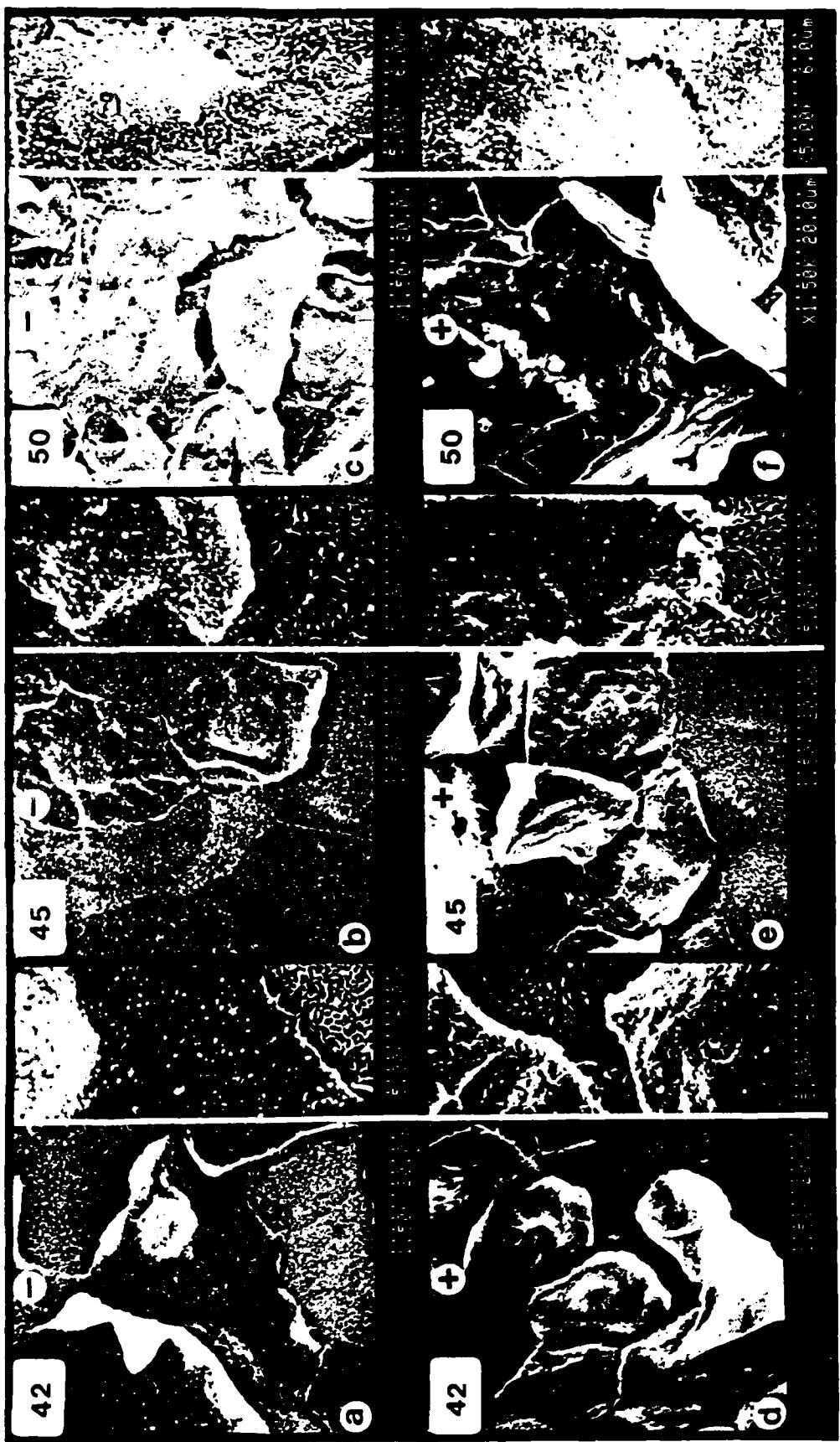


Fig. 4. Series of pictures showing changes in corneal epithelial cells visualized by SEM as temperature of incubation of corneas in M199 is increased from 42°C to 50°C.

Figure 5

Series of pictures showing by scanning electron microscopy, possible process of nucleus extrusion after incubation of cornea in phosphate buffered saline for 30 minutes at -5°C. In different epithelial cells, which can be seen successively in different figures: the barely visible nucleus is visible in figure (a) gradually swelling, in figure (b), (c) and (d) until it splits the corneal membrane(s) and floats free (N) from the cell, in figure (e). All figures at 1500X and the micron line is equal to 20 M.

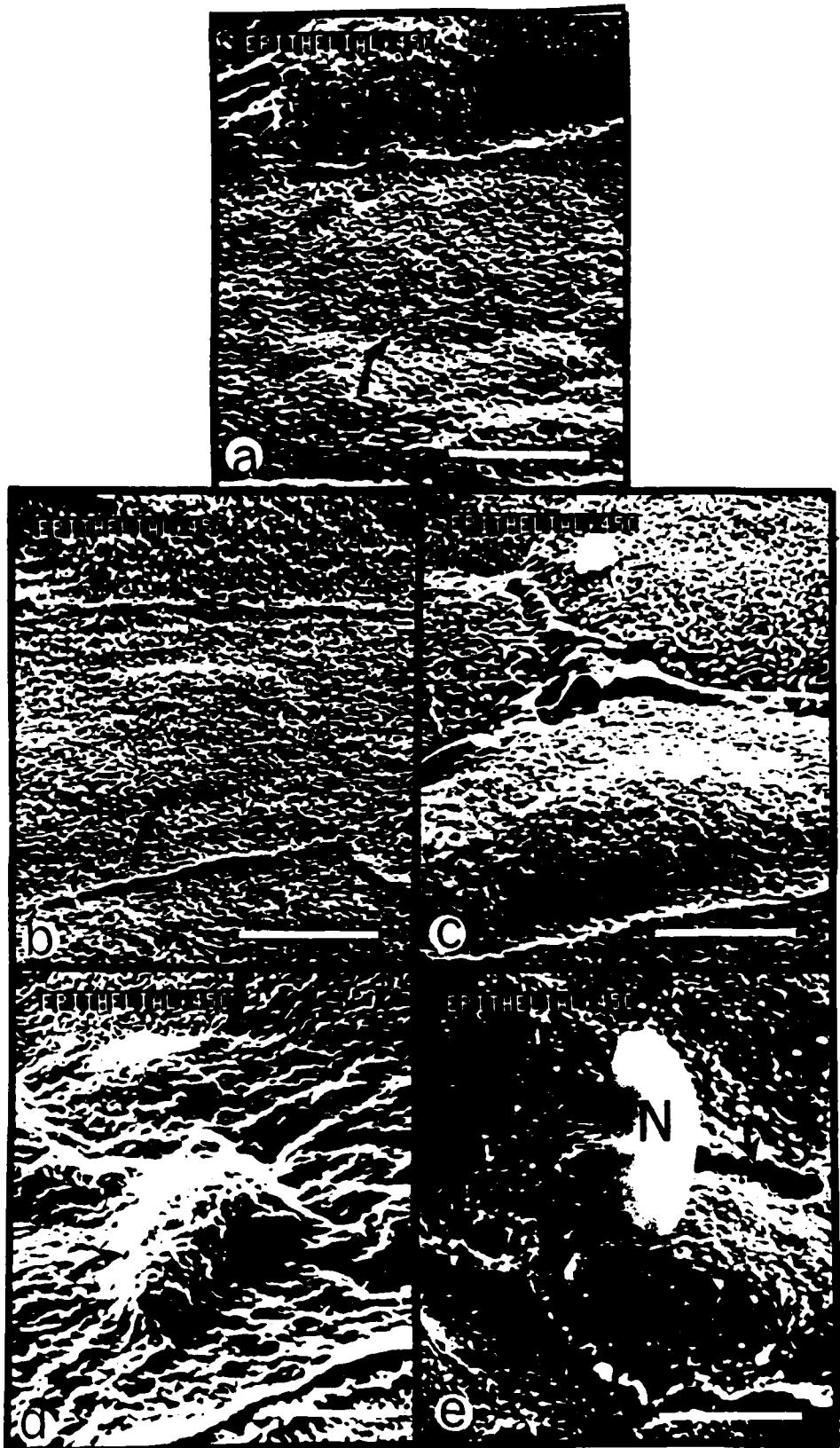


Fig. 5. Series of pictures showing by scanning electron microscopy, possible process of nucleus extrusion after incubation of cornea in phosphate buffered saline for 30 minutes at 45°C.

Figure 6

Series of pictures showing changes in corneal endothelial cells visualized by SEM as temperature of incubation of corneas in M199 is increased from 37°C to 40°C.

a),b),c) are of corneal endothelial cells incubated 30 min without vitamin E added to the medium.

d),e),f) are of corneal endothelial cells incubated 30 min with vitamin E (2.4 M) added to the medium.

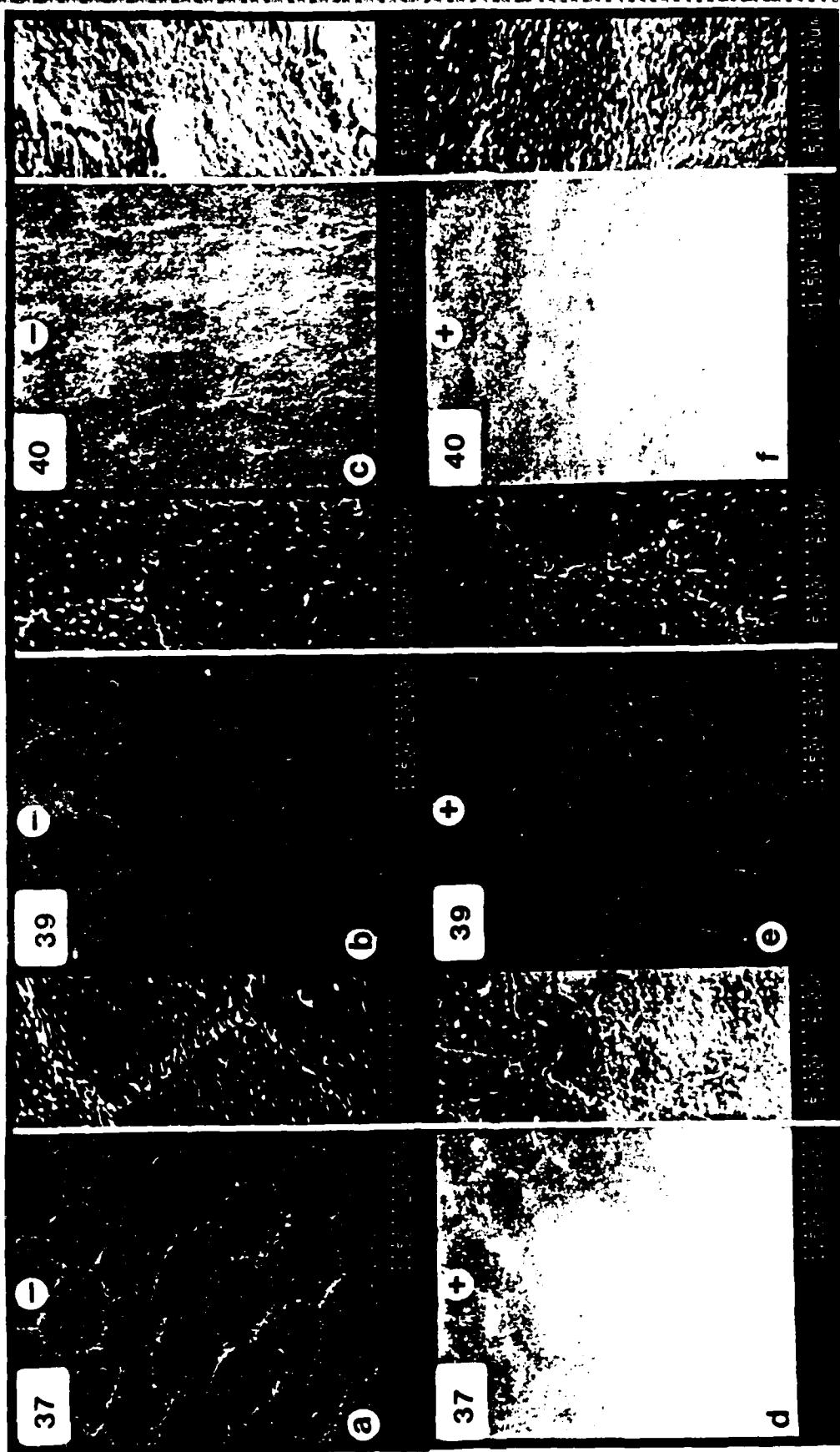


Fig. 6. Series of pictures showing changes in corneal endothelial cells visualized by SEM as temperature of incubation of corneas in M199 is increased from 37°C to 40°C.

Figure 7

Series of pictures showing changes in corneal endothelial cells visualized by SEM as temperature of incubation of corneas in M199 is increased from 42°C to 50°C.

a),b),c) are of corneal endothelial cells incubated 30 min without vitamin E added to the medium.

d),e),f) are of corneal endothelial cells incubated 30 min with vitamin E (2.4 M) added to the medium.

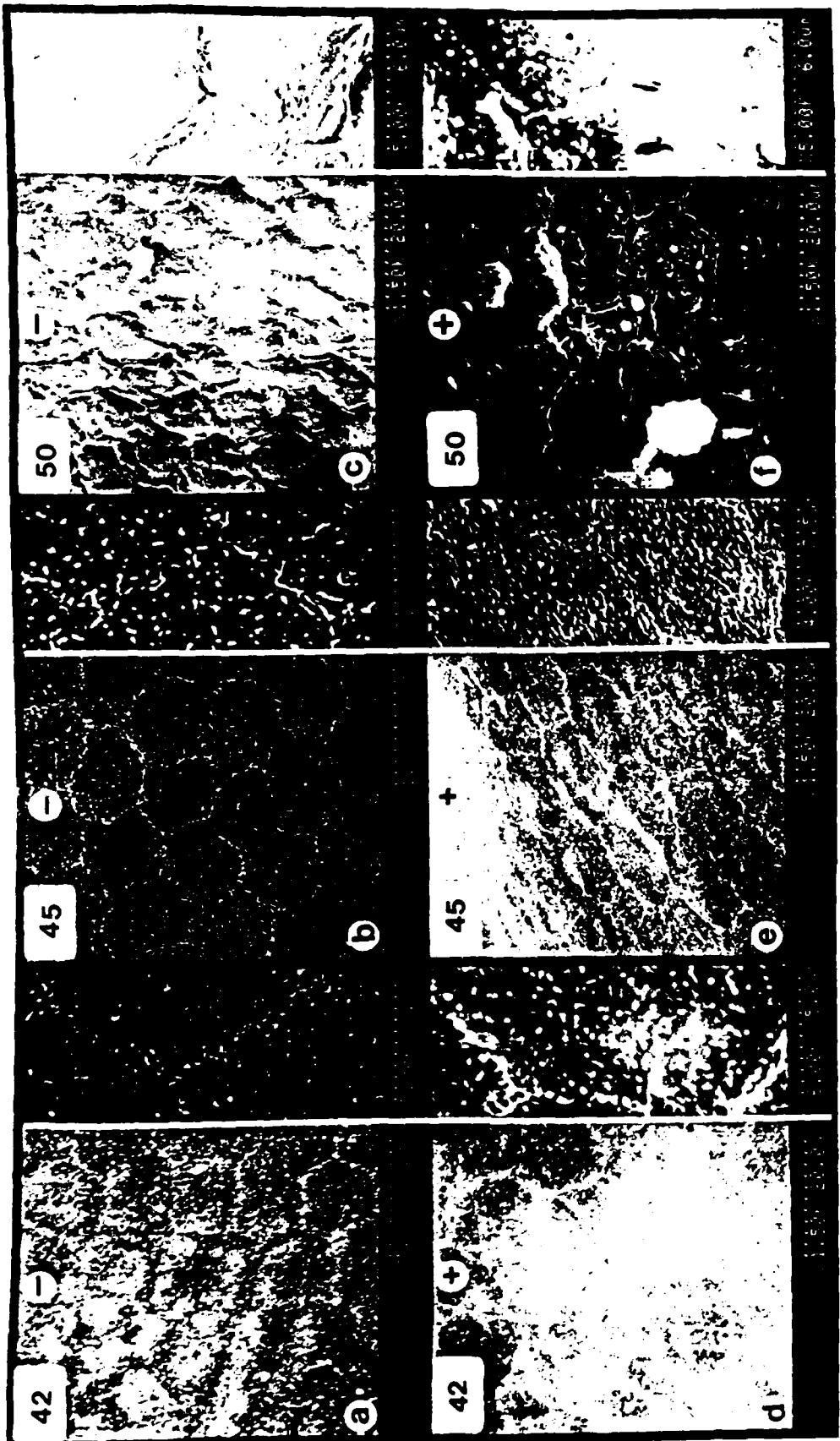


Fig. 7. Series of pictures showing changes in corneal endothelial cells visualized by SEM as temperature of incubation of corneas in M100 is increased from 42°C to 50°C.

Figure 8

Changes to stroma, visualized by SEM, at temperature of incubation of corneas in Medium 199 increases. All figures are at 7000X; the micron line is equal to 4.3 μ . Corneas were processed as described for Figure 1, above, sliced longitudinally prior to examination by SEM. Pictures show corneal stroma visualized after incubation at:

a) 3 $^{\circ}$ C

b) 39 $^{\circ}$ C

c) 42 $^{\circ}$ C

d) 50 $^{\circ}$ C

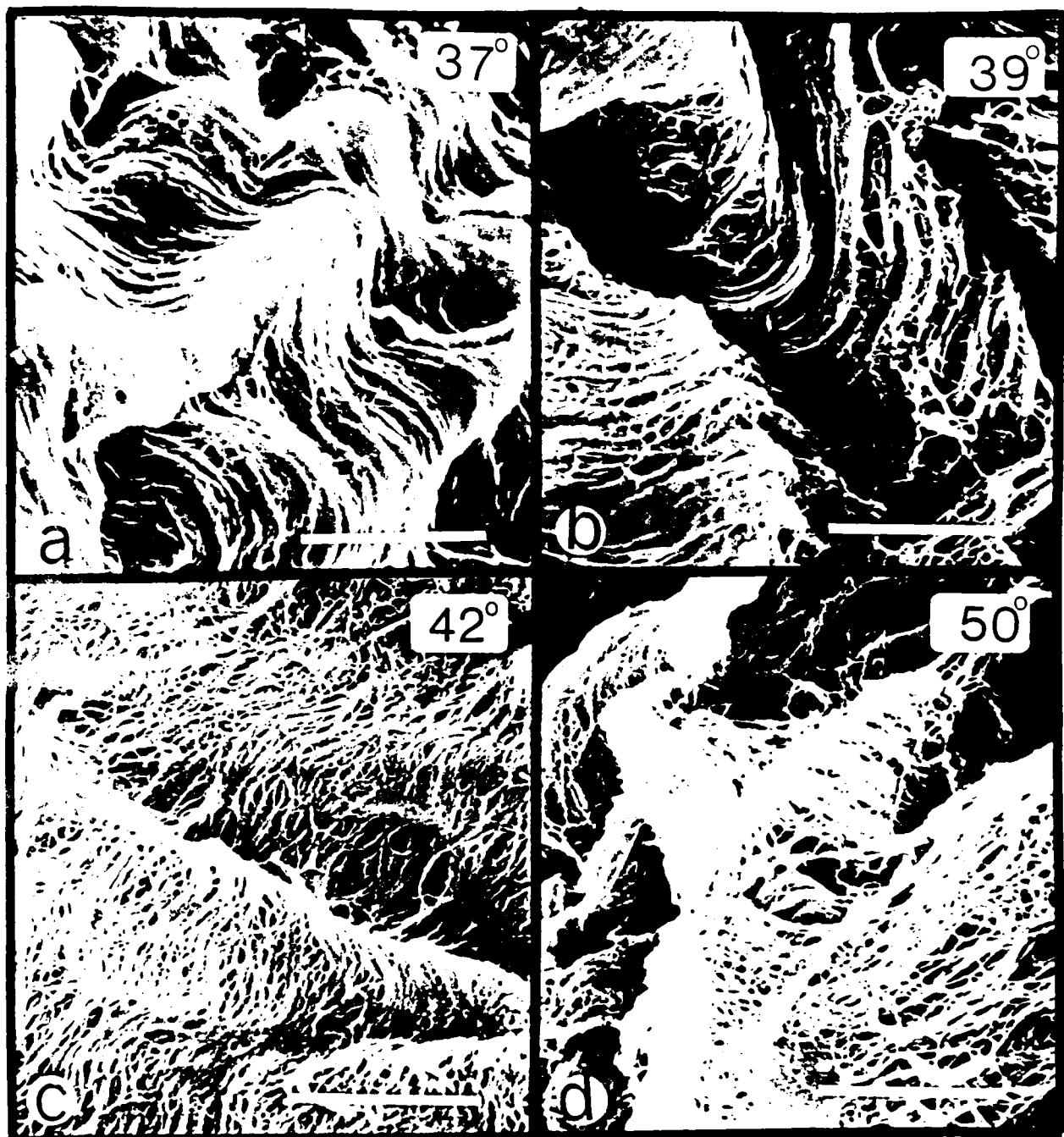


Fig. 8. Changes to stroma, visualized by SEM, at temperature of incubation of corneas in Medium 199 increase.

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